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# Direct Demonstration of Retroviral Recombination in a Rhesus Monkey

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**Recombination may be an important mechanism for increasing variation in retroviral populations. Retroviral recombination has been demonstrated in tissue culture systems by artificially creating doubly infected cells. Evidence for retroviral recombination in vivo is indirect and is based principally on the identification of apparently mosaic human immunodeficiency virus type 1 genomes from phylogenetic analyses of viral sequences. We infected a rhesus monkey with two different molecularly cloned strains of simian immunodeficiency virus. One strain of virus had a deletion in *vpx* and *vpr*, and the other strain had a deletion in *nef*. Each strain on its own induced low virus loads and was nonpathogenic in rhesus monkeys. When injected simultaneously into separate legs of the same monkey, persistent high virus loads and declines in $CD4^+$ lymphocyte concentrations were observed. Analysis of proviral DNA isolated directly from peripheral blood mononuclear cells showed that full-length, nondeleted SIVmac239 predominated by 2 weeks after infection. These results provide direct experimental evidence for genetic recombination between two different retroviral strains in an infected host. The results illustrate the ease and rapidity with which recombination can occur in an infected animal and the selection that can occur for variants generated by genetic recombination.**

Ten major subtypes of human immunodeficiency virus type 1 (HIV-1), represented by phylogenetic groups A through J, are currently recognized (21). Individual HIV-1 genomes that possess subgenomic regions that cluster with different subtypes have been discovered (3, 7, 9, 11, 19, 26). Such mosaic genomes have also been identified for HIV-2 and simian immunodeficiency virus (SIV) (10, 24, 25). It has been presumed that these mosaic structures arose through the process of genetic recombination (24, 25).

Recombination between two different strains of retrovirus can occur only when their RNA genomes are brought together into the same virus particle to create a heterozygous virion (16). Reverse transcription of heterozygous RNA genomes by a copy choice mechanism will produce genetic recombinants (16, 17, 28). A heterozygous virion is produced by the packaging of two different RNA molecules by a cell that is infected with two strains of virus. Although doubly infected cells have been generated artificially in cell culture systems, none have yet been identified in an infected host. Whether HIV-1 has ample opportunity for recombination in an infected host remains unclear. The uncertainty derives from questions regarding the numbers of cells harboring HIV-1 genetic information (1, 6, 23, 27), the timing and frequency of full expression of proviral genomes (22), the extent to which superinfection resistance will prevent a single infected cell from becoming infected with a second strain (2, 4, 13, 20), and the efficiency of the recombination process itself.

Evidence for the occurrence of recombination with HIV-1, HIV-2, and SIV is based on phylogenetic analyses of individual viral genomes (3, 7, 9–11, 19, 24–26) and thus is indirect in nature. It is not known whether mosaic viral genomes appeared as rare events in history and continue to be propagated in the population. Further, the time frame required to evolve into a different phylogenetic type is unknown. Retroviral recombination within an infected host has not to our knowledge ever been directly demonstrated. We thus designed an experiment that would readily detect SIV recombination in an infected monkey.

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## MATERIALS AND METHODS

**Isolation of DNA from peripheral blood mononuclear cells (PBMC).** For the isolation of DNA, cells were lysed in 0.6% sodium dodecyl sulfate and treated with 150 μg of proteinase K per ml at 56°C for 1.5 h. Three-tenths of a volume of saturated NaCl was added, and the solution was clarified by centrifugation. The NaCl step was repeated, and DNA was precipitated from the clarified supernatant with isopropanol. The DNA was washed with ethanol and air dried. Dried DNA pellets were resuspended in 179 μl of ultrapure, sterile $H_2O$ and incubated at room temperature overnight. Twenty microliters of 10× T4 DNA ligase buffer and 40 U of T4 DNA ligase were added, and the DNA suspensions were mixed gently by tapping. The ligation reaction mixture was incubated at room temperature for 1 h. Samples were stored finally at −20°C.

**PCR.** Double-nested PCR was performed with outer primers 5823VPX and 9816FEN3U in the first round and inner primers 5864VPX and 9788FEN3U in the second round. Primer sequences were as follows: 5823VPX, 5′-CAGGGAGAGAATCCCACCTGG-3′; 9816FEN3U, 5′-GAAGGCCTCTTGCGGTTAGCC-3′; 5864VPX, 5′-TAGGAGAGGCCTTCGAATGGC-3′; 9788FEN3U, 5′-AACCTCTTCCTCTGACAGGCC-3′. Each round of PCR consisted of 28 cycles of amplification with 5 μl of reaction mixture transferred from the first-round to the second-round reaction. Two units of rT*th* DNA polymerase XL was used for each reaction along with manufacturer's buffer (Perkin-Elmer, Foster City, Calif.). Other reaction components were 1.2 mM $Mg(OAc)_2$, 200 μM (each) deoxynucleoside triphosphates, 30 pm (each) primer, and 1 μg of genomic DNA template in a total volume of 100 μl. Round 1 amplification conditions consisted of a hot start (94°C, 1 min; 70°C, 2 min, during which enzyme was added), a two-step cycling profile (94°C, 15 s; 70°C, 5 min; 28 cycles), and a final incubation (72°C, 10 min). Round 2 amplification conditions were similar to those for round 1 with the exception of using 68°C in the two-step cycle profile instead of 70°C. All reactions were performed on a GeneAmp PCR System 9600 thermal cycler (Perkin-Elmer).

**Sequencing.** For direct sequencing of PCR-amplified DNA, three 100-μl reaction mixtures were combined and concentrated to a volume of 40 to 75 μl. Primers from PCR were removed by purification on CHROMA SPIN-400 col-

TABLE 1. Virological data

| Week no. | Virus load[a] | Antigenemia[b] (ng/ml) |
|---|---|---|
| 0 | NT[c] | <0.05 |
| 1 | 12,345 | <0.05 |
| 2 | 1,371 | 0.90 |
| 4 | 4,115 | <0.05 |
| 6 | 8,230 | <0.05 |
| 8 | 24,691 | NT |
| 12 | 12,345 | NT |
| 20 | 8,230 | NT |
| 27 | 8,230 | NT |

[a] Number of PBMC needed to isolate virus.
[b] SIV p27 antigen capture (Immunotech, West Brook, Maine).
[c] NT, not tested.

umns (Clontech Laboratories, Inc., Palo Alto, Calif.). Sequencing was performed with the Sequenase DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio). Sequencing primers were as follows: 6177VPX, 5′-TAAACC ACCTACCAAGGGAGC-3′; 6574XPV, 5′-CTCAAGGGTGTCTCCATGTC-3′; 9437NEF, 5′-GGAAGATGGATACTCGCAAT-3′; and 9759FEN, 5′-CTA AGATTCTATGTCTTCTTGC-3′.

## RESULTS

**Infection of the rhesus monkey.** A rhesus monkey was infected with two attenuated strains of SIV, Δvpxvpr and Δnef. Both are deletion mutants derived from the molecularly cloned parental strain SIVmac239, which causes AIDS in rhesus monkeys (18). The *vpxvpr* genes and the *nef* gene are required for full pathogenicity in rhesus monkeys. In the absence of *vpx* and *vpr*, or in the absence of *nef*, virus loads are low and AIDS does not develop (12, 18). The level of attenuation resulting from the *vpxvpr* deletion is similar to or slightly greater than that resulting from the deletion of *nef* (12, 18). Peak levels of the numbers of infectious cells in PBMC in the weeks immediately following infection are 20- to 1,000-fold lower with these mutants than with the parental SIVmac239. The numbers of infectious cells that persist for prolonged periods are also at least 100- to 1,000-fold lower with these mutant viruses than with the parental SIVmac239 (12, 18).

The rhesus monkey was inoculated intravenously in one leg with Δvpxvpr virus containing 154 ng of p27 and in the other leg with Δnef virus, also containing 154 ng of p27. The same stocks of Δvpxvpr and Δnef viruses have been used previously to infect rhesus monkeys without any evidence of high virus loads, disease progression, or wild-type virus (8, 12, 18). Blood samples were obtained at various times after inoculation and used to monitor several parameters. A spike of p27 antigenemia corresponding to 0.9 ng/ml was detected in plasma 2 weeks after inoculation (Table 1). Such a spike has always been detected at week 2 with wild-type parental SIVmac239 but not with either of the deletion mutants used (12, 18). High cell-associated virus loads, measured as the frequency of infectious cells in PBMC (8, 12, 18), were induced and maintained in this animal, similar to the pattern obtained with the parental SIVmac239 (30) but dissimilar to the pattern obtained with either of the mutant viruses (8, 12, 18) (Table 1). Furthermore, the frequency of $CD4^+$ cells in PBMC decreased from 51 and 52% at weeks 1 and 0, respectively, to 33% at week 12 and 27% at week 28 (data not shown).

**Amplification of proviral DNA.** The above results suggested that a recombination event had taken place between the two viruses to yield nondeleted, parental virus. To examine this possibility, total cell DNA was prepared from PBMC isolated at 2 and 6 weeks postinfection, and proviral sequences were amplified by PCR.

In order to provide precise size standards, CEMx174 cell cultures (15) were separately infected with the full-length parental virus SIVmac239, deletion mutant Δvpxvpr, and deletion mutant Δnef. Total cell DNA was prepared by the same DNA preparation method that was used for the PBMC. For negative controls, DNA was prepared from uninfected CEMx174 cells and from uninfected rhesus monkey PBMC. PCR primers that span a DNA sequence of approximately 4 kb that encompasses both the *vpxvpr* and *nef* deletions were designed. PCR amplification with DNA from PBMC samples from the infected monkey yielded a fragment of approximately 4 kb (Fig. 1). When subjected to the same double-nested PCR procedures in parallel in multiple independent experiments, both of the negative DNA samples were completely devoid of amplified bands, indicating the absence of PCR contamination. A representative CEMx174 DNA control is shown in Fig. 1.

**Restriction enzyme analysis of amplified proviral DNA.** The entire region spanning both the *vpxvpr* and *nef* deletions had to be amplified in one piece in order to detect the presence of recombinant sequences. Since the sizes of the *vpxvpr* and *nef* deletions were 265 and 182 bp, respectively, their presence could not be established unambiguously from the sizes of the amplified fragments in the gel shown in Fig. 1. The ~4-kb amplified fragments were therefore digested with restriction enzymes *Sph*I and *Sst*I and were further analyzed by agarose gel electrophoresis.

The *Sph*I enzyme cut is diagnostic for the presence of the *vpxvpr* deletion. Restriction of the ~4-kb PCR product with *Sph*I yielded the expected fragments of 3,386 and 628 bp for full-length SIVmac239 and fragments of 3,386 and 363 bp for the Δvpxvpr mutant; the presence of the *vpxvpr* deletion can thus be determined by the size of the smaller *Sph*I fragment: 363 versus 628 bp. When amplified DNAs from the week 2 and week 6 PBMC samples were cut with *Sph*I, only the 628- and 3,386-bp fragments were detected. The sizes of the *Sph*I fragments were identical to those of the full-length, parental SIVmac239 and clearly distinct from those of the Δvpxvpr control (Fig. 2A).

Similarly, the *Sst*I enzyme cut is diagnostic for the presence

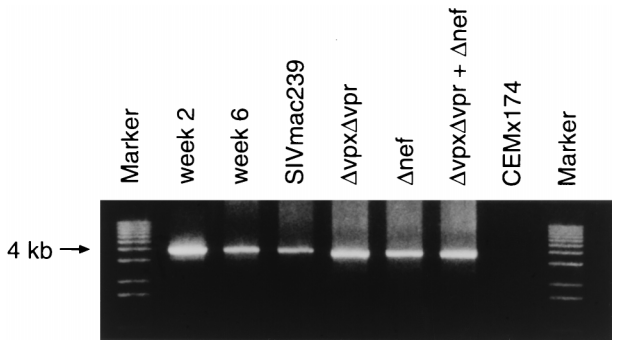


FIG. 1. PCR amplification of SIV sequences. One microgram of total cell DNA was used for amplification by double-nested PCR. The products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Lanes labeled week 2 and week 6 represent total cell DNA prepared from PBMC isolated at 2 and 6 weeks, respectively, after coinfection of the rhesus monkey. The remaining lanes show the products from total cell DNA prepared from CEMx174 cells infected with the following viruses: (i) SIVmac239, full-length parental virus; (ii) Δvpxvpr, attenuated SIVmac239 with deletions in *vpx* and *vpr*; (iii) Δnef, attenuated SIVmac239 with a deletion in *nef*; (iv) Δvpxvpr plus Δnef, a mixture of 0.5 μg each of total cell DNA prepared from cells infected separately with Δvpxvpr and Δnef viruses; (vi) CEMx174, uninfected cells.

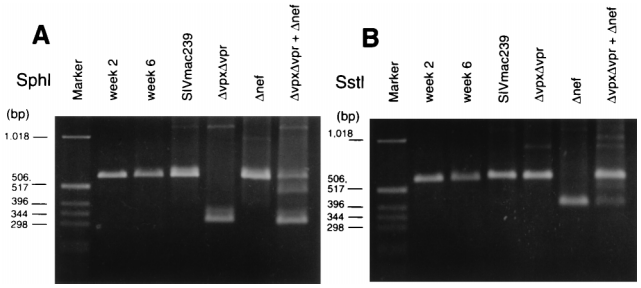


FIG. 2. Restriction enzyme analysis of PCR-amplified SIV DNA. The ~4-kb fragments shown in Fig. 1 were digested with *Sph*I (A) and *Sst*I (B). *Sph*I is diagnostic for the presence of the *vpxvpr* deletion, while *Sst*I is diagnostic for the presence of the *nef* deletion. See the legend to Fig. 1 for a definition of lanes.

of the *nef* deletion. Restriction of the ~4-kb PCR product with *Sst*I yielded the expected fragments of 3,408 and 606 bp for the full-length SIVmac239 and fragments of 3,408 and 424 bp for the *nef* mutant; the presence of the *nef* deletion can thus be determined by the size of the smaller *Sst*I fragment: 424 versus 606 bp. The sizes of the *Sst*I fragments for weeks 2 and 6 were identical to those of the full-length, parental SIVmac239 and clearly distinct from those of the Δnef control (Fig. 2B). These results demonstrate that only full-length, recombinant SIVmac239 was detected at weeks 2 and 6 after coinfection of the rhesus monkey.

**Controls for PCR recombination.** In order to rule out the possibility that the observed results were due to selective PCR recombination, 0.5 μg of total cell DNA prepared from cells infected individually with the Δvpxvpr and Δnef mutants was mixed and amplified alongside the other samples in double-nested PCR (Fig. 1). Restriction enzyme analysis of the ~4-kb PCR product with *Sph*I and *Sst*I showed a mixture of the two deletion mutants (Fig. 2; representative of six independent experiments). These results indicate that the full-length, recombinant SIVmac239 observed at weeks 2 and 6 in the coinfected rhesus monkey was not the result of PCR recombination.

**Sequencing of recombinant proviruses.** The PCR-amplified DNAs from PBMC taken at weeks 2 and 6 were directly sequenced. Primers were located upstream and downstream of the regions corresponding to the *vpxvpr* and *nef* deletions. The sequence of the PCR-amplified DNA matched exactly that of the full-length, parental SIVmac239; no changes were found within either of the regions. Therefore, the observed restoration to full-length size (Fig. 1 and 2) was not due to insertion of another sequence, duplication, or DNA rearrangement.

## DISCUSSION

The studies described in this report provide direct experimental evidence for genetic recombination between two SIV strains in an infected animal. A rhesus monkey was inoculated with two different strains of SIV, and recombinant virus was found to predominate by 2 weeks postinfection. Recombinant virus was strongly selected in these experiments because both of the starting strains were significantly impaired in their ability to replicate in monkeys.

Recent studies of HIV-1-infected individuals reveal that the long asymptomatic phase of infection is not a latent period but is a time of high activity for the virus (14, 29). It has been estimated that the minimum daily production of HIV-1 particles in infected individuals is on the order of $10^9$ virions per day (14). It has also been estimated that HIV-1 replicates at a rate of at least 300 cycles per year, resulting in an estimated 3,000 generations during the average 10-year period of infection (5). Despite the availability of this highly quantitative information on viral dynamics, assessment of the opportunity for HIV-1 recombination in an infected host is unfortunately not possible. Uncertainty over the numbers of cells replicating HIV-1 genetic information at any one time (1, 6, 23, 27) and the efficiency of superinfection resistance prevent any such calculation of the likelihood of recombination events.

The rhesus monkey used for this study can be estimated to contain approximately $10^{11}$ total body lymphocytes. The numerical odds of obtaining 10 doubly infected cells at peak virus load would require only 1 in $10^5$ cells replicating each deletion mutant virus. This theoretical calculation does not take into account the unknown contribution of superinfection resistance or the possibility of localized replication in discrete areas of lymphoid tissue. Nonetheless, our results indicate that there is ample opportunity for genetic recombination even with these attenuated mutants. The opportunity for recombination with wild-type viruses, which replicate to much higher levels than the strains used in our study, is expected to be considerably greater. Recombinants with a selective advantage, such as that described in the results reported here, could significantly impact the progression of events in vivo.

Our studies with SIV demonstrate not only that AIDS virus recombination can occur readily in an individual but also that recombinants can be selected rapidly by biological forces. Two weakly replicating SIVs recombined in a coinfected rhesus monkey to yield a fully replication-competent virus with pathogenic potential. Recombination must be viewed as a viable mechanism for increasing AIDS virus variation in infected individuals.

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